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Abstract
Vaccine adjuvants are chemicals, microbial components, or mammalian proteins that enhance the immune response to vaccine antigens. Interest in reducing vaccine-related adverse effects and inducing specific types of immunity has led to the development of numerous new adjuvants. Adjuvants in development or in experimental and commercial vaccines include aluminum salts (alum), oil emulsions, saponins, immune-stimulating complexes (ISCOMs), liposomes, microparticles, nonionic block copolymers, deriv-atized polysaccharides, cytokines, and a wide variety of bacterial derivatives. The mechanisms of action of these diverse compounds vary, as does their induction of cell-mediated and antibody responses. Factors influencing the selection of an adjuvant include animal species, specific pathogen, vaccine antigen, route of immunization, and type of immunity needed.

Keywords
Enhancement of immunity, Immunization, Immunomodulation

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Review

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Vaccine adjuvants are chemicals, microbial components, or mammalian proteins that enhance the immune response to vaccine antigens. Interest in reducing vaccine-related adverse effects and inducing specific types of immunity has led to the development of numerous new adjuvants. Adjuvants in development or in experimental and commercial vaccines include aluminum salts (alum), oil emulsions, saponins, immune-stimulating complexes (ISCOMs), liposomes, microparticles, nonionic block copolymers, derivatized polysaccharides, cytokines, and a wide variety of bacterial derivatives. The mechanisms of action of these diverse compounds vary, as does their induction of cell-mediated and antibody responses. Factors influencing the selection of an adjuvant include animal species, specific pathogen, vaccine antigen, route of immunization, and type of immunity needed.

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The advent of vaccination in the 1800s had unprecedented benefits for both human and veterinary medicine. Vaccines now control or prevent numerous human and animal diseases, including scourges such as poliomyelitis, smallpox, distemper, and parvovirus enteritis. The contributions of pioneer vaccine researchers such as Pasteur and Jenner are well known. Less often noted are parallel discoveries that made vaccination more powerful and, indeed, in some cases, even feasible. In the 1920s, Ramon[1,2] discovered that horses that developed abscesses at the injection site had higher antibody titers after vaccination. Subsequently, he and others found that titers could be enhanced by injections of tapioca, agar, lecithin, saponin, and aluminum compounds.[2,3] In the 1930s, Freund et al[4] invented a particularly effective combination of mineral oil, water, and killed mycobacteria. These discoveries were the basis for the development of adjuvants, vaccine additives that boost immunity and alter immune responses to coadministered antigens.

A protective immune response must enhance those aspects of immunity that will be effective against specific pathogens. Vaccines against extracellular bacteria should induce immunoglobulin G (IgG) to opsonize the bacteria for phagocytosis, to activate complement, and to neutralize toxins. On mucosal surfaces, IgA prevents attachment, and IgE arms mast cells under the mucosal surface to react if the pathogen invades. Other pathogens are effectively destroyed only by cell-mediated immune reactions. For facultative intracellular bacteria (eg, *Mycobacteria, Brucella,* and some *Salmonella*), activated macrophages are required. If a pathogen such as a virus, *Listeria,* or some protozoan parasite begins to replicate in the cytoplasm of a cell, a cytotoxic T-lymphocyte (CTL) response is needed to destroy the infected cell. Often, a combination of immune mechanisms may be the most effective defense: for example, an IgA response to inhibit viruses from invading through a mucosal surface, an IgG response to neutralize viruses that do invade, and a cytotoxic T-cell response to destroy cells that the virus manages to infect.

Infection with a virulent pathogen usually provides the necessary signals to induce the correct type or types of immune response. By mimicking the virulent organism, modified live vaccines also can provide these signals. Although they often are very effective, these vaccines have several potential disadvantages and are undesirable for some diseases.[5] The main alternative at present is a killed vaccine. However, killed vaccine antigens administered by an unnatural route of exposure (ie, injection) may not provide the signals necessary to induce protective immunity. Less purified killed vaccines sometimes contain bacterial or viral components that can serve as ''built-in'' adjuvants, but more purified antigens usually do not stimulate a strong and lasting immune response.[6] This is particularly true for highly purified peptides or carbohydrates. In the absence of adjuvants, such killed antigens may even result in tolerance.[7] Adjuvants can provide artificial signals to the immune system to initiate the immune response. By doing so, adjuvants minimize the number of immunizations necessary for a good immune response. They also may decrease the amount of antigen needed, making the vaccine more cost-effective. Some but not all adjuvants also can shift responses toward the more effective form or forms of immunity.

When developing a vaccine, it is essential to know what type of immune response will provide optimal protection and then select an adjuvant that will help induce that type of immune response without unacceptable adverse effects. Considerable trial and error often is needed to find a safe and effective adjuvant for a particular pathogen in a given species. Induction of the wrong type of immune response actually can enhance disease pathogenesis after the animal becomes exposed to the pathogen. Early attempts to develop a vaccine against infectious peritonitis virus in cats are an example of this response.[8] Host factors also influence the effectiveness of the adjuvant. Whereas young, healthy

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individuals sometimes can mount an effective immune response to a less than ideal vaccine, those with poor immunity may not. Individuals with poor immunity include the immunocompromised, the very old, and the very young. By boosting immune responses, adjuvants can allow vaccines to be used effectively in these other groups.[9]

Mechanisms of Action

Most adjuvants are chemicals, microbial components, or mammalian proteins. Despite years of research, their mechanism of action remains somewhat speculative.[7,10] In general, most appear to enhance antigen presentation, improve antigen stability, or act as immunomodulators.[9] A single adjuvant may have more than 1 mechanism of action. For example, adjuvants that help preserve the antigen's structure can improve the effectiveness of the vaccine and also increase its shelf life.[10]

Adjuvants that influence antigen presentation can affect this complex process at numerous points. During an immune response, vaccine antigens must reach secondary lymphoid tissues, usually the lymph nodes. Most of these antigens are carried to lymph nodes by dendritic cells. These antigen-presenting cells (APCs), as well as macrophages and B cells, process the antigens and show epitopes to T cells in major histocompatibility complex (MHC) molecules. Dendritic cells, macrophages, and B cells also provide other signals needed to initiate immunity, such as costimulation by the B7 family of molecules. Any adjuvant that can improve antigen uptake by these cells, increase costimulatory or MHC molecules, or increase the cell's migration to the lymph nodes can improve immunity. Some adjuvants appear to trap the antigen at the injection site and provide a continuing supply to local APCs. This depot effect may reduce elimination of the antigen by the liver.[10] Oil emulsions such as Freund's adjuvants can form short-term (8–10 days) depots that are sufficient to enhance immunity.[10] Newer microparticle adjuvants can form long-term (1–6 months) depots and may be able to deliver pulsed doses of antigens.[10,11] Other adjuvants may work by saturating Kupffer cells in the liver.[10] By reducing hepatic uptake of the antigen, such adjuvants may increase the amount of antigen reaching the APCs. This mechanism has been suggested for the derivatized polysaccharide adjuvants, including high-molecular-weight sulfated dextrans and diethylaminoethyl (DEAE) dextran.[10]

Some adjuvants improve antigen targeting to APCs.[9,10] Particulate adjuvants such as aluminum salts (alum) promote formation of aggregates; these aggregates are more easily phagocytosed.[10] Carbohydrate polymers such as mannan or acemannan may be able to guide antigens to APCs by attaching to carbohydrate receptors.[10] Carrier proteins such as bovine serum albumin, keyhole limpet hemocyanin (KLH), and diphtheria or tetanus toxoid can aid the presentation of haptens or carbohydrate antigens by recruiting T-helper cells. Some adjuvants also appear to target the antigen to specific compartments of the APC and influence the induction of a CTL response. T-helper cells and CTL responses are activated when they interact with antigens presented in MHC II or MHC I molecules, respectively. Although some crossing of antigens (''cross-presentation'') occurs between pathways, antigens presented in MHC II molecules usually come from outside the APCs and are taken up by phagocytosis, whereas antigens presented in MHC I molecules generally originate in the cytoplasm of the APCs.[12,13] Most adjuvants can effectively stimulate T-helper cells and humoral immunity. Some, such as liposomes, also appear to deliver antigens to pathways that lead to the presentation in MHC I molecules and the induction of a CTL response.[9,10,14] In some cases, cross-presentation may be important in generating CTLs.

Immunomodulation is another mechanism of action. Immunomodulators stimulate the immune system by altering the cytokine network.[10,11] Some adjuvants up-regulate cytokines and the immune system in general. More often, adjuvants influence the type of immunity by enhancing some cytokines and reducing the concentrations of others. The cytokines interferon gamma (INF-γ), interleukin-2 (IL-2), and IL-12 are associated with T-helper cell type 1 (Th1) responses and cell-mediated immunity (CMI). IL-4, IL-5, IL-6, IL-13, and possibly IL-10 are associated with T-helper type 2 (Th2) responses and humoral immunity. By shifting the balance of these 2 sets of cytokines, adjuvants such as saponins may stimulate CMI to an antigen that would normally induce only antibodies.[9,10,15] Some immunomodulatory adjuvants increase the expression of costimulatory molecules or MHC molecules on APCs, either directly or by induction of cytokines.[7,16–18]

Recently, attempts have been made to fit adjuvant mechanisms into more general hypotheses of immune function. According to some current hypotheses, all APCs 1st must be activated for antigen presentation before they can initiate immune responses. In one model, this activation is thought to occur when pattern recognition receptors on the APCs bind to conserved motifs in bacterial lipopolysaccharides, sugars, or other moieties.[7,19] If this hypothesis is correct, adjuvants may act by mimicking these primitive bacterial signals.[7] In fact, many adjuvants are derivatives of bacteria or resemble the motifs in bacterial proteins, carbohydrates, or DNA. However, this model may not explain adjuvants such as oil emulsions, saponins, or alum.[7] According to a 2nd model (the danger model), APCs recognize endogenous signals released by damaged, stressed, or dying cells.[7,19] Gallucci et al[19] have found that necrotic fibroblasts or blood vessels can act as very effective adjuvants. In addition, an influx of neutrophils can be seen after vaccination with some adjuvants.[7] The danger model, however, does not seem to account for the effects of adjuvants such as liposomes.[7]

Adverse Effects and Potential Hazards of Adjuvants

When immune responses destroy invading microorganisms, they cause tissue damage and result in some of the clinical signs of illness. Similarly, as agents that enhance immune responses, adjuvants can increase the adverse effects of the vaccine. These adverse effects are influenced by the interactions of the specific adjuvant and antigen.[9,20] Systemic, nonspecific adverse effects can include fever, arthritis, uveitis, anorexia, soreness, and lethargy.[9,20,21] Theoretically, adjuvants also may increase the probability of au-

toimmune reactions. Overdoses of IL-2, a cytokine proposed as an adjuvant, have been linked to autoimmune diseases.[22] Autoantibodies have been detected after vaccination with typical canine distemper, rabies, and parvovirus vaccines, and a temporal association has been noted between autoimmune hemolytic anemia and vaccination in dogs.[21] Adjuvants also may have specific adverse effects related to their chemical nature. For example, some crude saponin adjuvants can result in hemolysis if injected IV.[23]

More often, adjuvants cause local reactions including inflammation and, more rarely, granulomas or sterile abscesses. In dogs, the vaccines most often associated with local reactions are rabies or distemper combinations; in cats, rabies vaccines most often are linked to local non-neoplastic reactions.[21] Although most of these reactions are minor and transient, 3 serious considerations emerge. First, severe inflammation can trap antigens at the injection site and prevent them from being recognized by the immune system.[22] Second, some adjuvants can result in carcass trim losses in food animals.[5,24] Certain alum-containing vaccines, for example, can cause large granulomas in sheep.[24] Granulomas are associated particularly with depot adjuvants and can take weeks or months to resolve.[21] Finally, local inflammation and granulomas after vaccination have been linked to the development of vaccine-associated sarcomas in cats.

In the late 1980s, an alarming increase in the incidence of vaccine-associated sarcomas in cats was observed. Currently, the incidence of these sarcomas is thought to be 1–10 per 10,000 vaccinated cats.[25] These vaccine-associated sarcomas occur at the site of vaccination, sometimes contain residual aluminum adjuvant, and have features in common with inflammatory reactions.[26] They have been linked to rabies, feline leukemia (FeLV), and other vaccines, as well as to injections of nonvaccine products.[25,27] The exact role of antigens, adjuvants, or other factors in sarcoma development remains to be determined, but circumstantial evidence suggests that adjuvants may be involved, and the timing of sarcoma development is suspicious. Sarcomas became more common as adjuvanted vaccines became more common. In the 1980s, the 1st FeLV vaccines had reached the market, and modified live rabies vaccines were being replaced by killed adjuvanted vaccines.[25] Adjuvants also increase inflammation, which seems to be an important risk factor for these sarcomas.[25,28–30] Apparently, some vaccines result in inflammatory granulomas, which can, in a few cats, develop into cancer. Aluminum adjuvants can cause inflammation, and some authors suggest they should be avoided in cats.[31] However, this recommendation is controversial. Not only have adjuvants other than aluminum been linked to sarcomas, but tumors also can occur when vaccines without adjuvants are used.[24,26,31]

At present, no specific brands of vaccines seem to be associated with sarcomas.[25] In general, it can be difficult to establish the incidence of rare adverse effects for any particular vaccine. Although vaccines for veterinary use must be labeled with the adverse effects seen during premarket testing, veterinary vaccine manufacturers are not routinely required to update labels with postmarketing adverse effects or to record reports of adverse effects received from veterinarians.[21] There may even be a disincentive to routinely list postmarketing adverse effects, because veterinarians may assume incorrectly that vaccines with more listed adverse effects are more dangerous.[21] In January 2002, the US Department of Agriculture (USDA) proposed a new rule that would make it mandatory for manufacturers to keep a record of adverse effects and report them to the USDA.[32]

Although these potential hazards must be appreciated, killed vaccines generally are considered safer than modified live vaccines, which have the potential to induce disease in immunocompromised animals.[5,6] In most instances, the adverse effects of adjuvants are mild, and in general, their benefits outweigh the hazards of their use. In specific situations such as vaccinations of cats, the benefit versus hazard equation may be different and may need to be considered more carefully.

Major Types of Adjuvants

Since the discovery of the 1st adjuvants in the 1920s, hundreds of substances with adjuvant activity have been found. Several of the adjuvants discovered by Ramon,[1] Glenny et al,[3] and Freund et al[4] continue to be used. Until recently, alum compounds were the only adjuvants allowed in vaccines for humans. Both alum and oil emulsions were used in vaccines for animals. Freund's original emulsion, called Freund's complete adjuvant (FCA), was abandoned because of toxicity, but Freund's incomplete adjuvant (FIA), which contains no mycobacteria, still is used sometimes when a strong adjuvant is needed and inflammation is not an important drawback. Recently, there has been an upsurge of interest in new adjuvants that can induce CMI or elicit more effective immune responses with fewer adverse effects. Some vaccines contain proprietary adjuvants whose composition cannot be made public.

Alum and Calcium Salts

Alum and calcium salts are relatively weak adjuvants that mainly induce Th2 responses and few if any CTLs.[6,10,15] Calcium salts rarely are used, but alum is widespread in vaccines for human and veterinary use. The amount of alum varies with the vaccine. For example, studies by Macy[24] indicate that 1-year killed rabies vaccines generally contain less alum than 3-year products. However, 3-year rabies vaccines sometimes are marketed as 1-year vaccines.[24] Multiple injections often are necessary for long-lasting immune responses, and titers often are lower than those observed with other adjuvants. Alum and calcium salt adjuvants once were thought to form antigen depots, but this assumption has been questioned.[7] Alum adjuvants but not calcium salt adjuvants also are immunomodulators.[10] The safety of these adjuvants is thought to be excellent.[6,10] Serious adverse effects are rare, but allergic reactions and granulomas occasionally are seen.

Oil Emulsion Adjuvants

Oil emulsion adjuvants contain a mixture of oil and aqueous phases, stabilized by a surfactant. Without other components, oil-based adjuvants stimulate mainly antibody responses, but under some circumstances, water-in-oil emulsions may be able to activate CTLs.[10,11,15,20,33] Induction of

either Th1 or Th2 cytokines is weak to nonexistent.[11,15] In general, oil emulsions are stronger adjuvants than alum, but at the cost of increased injection-site reactions and granulomas. Traditionally, these adjuvants contained mineral oil, but there is a risk that this oil is contaminated by carcinogenic polycyclic aromatic hydrocarbons; consequently, many emulsions now contain vegetable or animal oils such as shark liver oil and squalene. Adjuvants that contain metabolizable oils have a better safety record than adjuvants based on mineral oil and tend to induce only weak inflammation.[10,20] Unfortunately, the increase in safety can be accompanied by a decrease in efficacy.[20]

The 3 types of oil-based adjuvants are water-in-oil emulsions, oil-in-water emulsions, and water-in-oil-in-water emulsions. Water-in-oil emulsions such as FIA contain microdroplets of an aqueous phase in an oil, stabilized by a surfactant. These adjuvants release the vaccine antigens slowly and can give good long-term immunity.[20] Injection-site reactions are fairly common with water-in-oil adjuvants. These emulsions also are viscous, which can make them difficult to inject. Because of these disadvantages, water-in-oil emulsions are not used in vaccines for humans and companion animals. They can be found in some ruminant, poultry, and fish vaccines and sometimes are used in research animals. Oil-in-water emulsions such as MF59 contain microdroplets of oil in water, stabilized by surfactants. Oil-in-water emulsions free the antigen quickly and give good short-term immunity.[20] The droplets of oil may be able to carry antigens to lymph nodes in lymphatic vessels, and antigen depots may form on APCs in the lymph nodes rather than at the injection site.[33] Oil-in-water emulsions are less viscous and less likely to promote inflammation than water-in-oil formulations.[24] An influenza vaccine containing an oil-in-water adjuvant has been registered for use in humans in Italy. Water-in-oil-in-water emulsions contain microdroplets of water in an oil that is dispersed through an aqueous phase. These adjuvants release antigens more quickly than water-in-oil emulsions but more slowly than oil-in-water emulsions.[20] Water-in-oil-in-water emulsions can promote both short- and long-term immunity and are less viscous than water-in-oil emulsions.[20,24] Earlier formulations were not very stable, but newer techniques appear to have overcome this problem.[20]

Liposomes and Archaeosomes

Liposomes are vesicles of cholesterol and phospholipids that resemble crude cell membranes. These adjuvants can incorporate antigens either within the lumen or in the membrane. They can induce humoral immunity and, in some cases, activate CTLs.[9,10,23] Although liposomes appear to fuse with endosomes to enter the MHC II pathway, large quantities of some epitopes seem to spill into the cytoplasm.[14] Liposomes have been used for years as vehicles to deliver drugs and have a good safety record.[10,14] Immunomodulators sometimes are added to increase their efficacy but can also increase adverse effects. A hepatitis A vaccine for humans with a liposomal adjuvant recently was licensed.

Archaeosomes, liposomes made with lipids from the Archaea, appear to be particularly good adjuvants. The Archaea are unusual bacteria-like organisms that seem to belong to a separate domain (or empire) that is distinct from both bacteria and eucaryotes. The Archaea often thrive in extreme environments and contain unusual lipids that can form particularly stable liposomes.[34] This stability may contribute to the good memory responses to incorporated antigens.[34] Whereas lipids from species of Archaea vary in their effectiveness, some can induce much higher titers than alum.[34] Some lipids also seem to be immunomodulators.[34] Archaeosomes can induce both Th1 cytokines (INF-γ) and Th2 cytokines (IL-4), as well as cell-mediated responses to several antigens.[34] The safety of archaeosomes still is being evaluated, but to date, no important adverse effects have been seen.[34] This lack of adverse effects is not entirely unexpected, because some Archaea can be found in mammalian hosts. *Methanobrevibacter smithii*, one organism used to make archaeosomes, is a normal resident in the gastrointestinal tract of humans.

Nanoparticles and Microparticles

Nanoparticles and microparticles are tiny solid particles made from biodegradable polymers, especially cyanoacrylates and poly(lactide-co-glycolide) copolymers. Nanoparticles (10–1,000 nm) differ from microparticles (1–100 μm) only in their size. The polymers used in these adjuvants also are used as suture material, prostheses, and drug carriers and are thought to be nontoxic. In preliminary studies, no serious adverse effects have been observed.[35] One unique characteristic of these adjuvants is their ability to form a long-term depot that can release antigen for up to several months. Mixtures of fast-releasing and slow-releasing microparticles theoretically could provide both primary and booster immunizations with 1 injection.[6,10,11,15] In rats, 1 dose of tetanus toxoid with a microparticle adjuvant gave immunity comparable to 3 doses with alum.[36]

Microparticles can induce CMI, including CTLs, as well as humoral immunity.[6] They usually are not immunomodulators, but immunomodulators can be incorporated to improve their effectiveness.[11] Microparticle adjuvants can protect incorporated antigens from harsh conditions such as low pH, bile salts, and enzyme activities. For this reason, they may be particularly useful in oral and intranasal vaccines. Technical problems in manufacture can be a disadvantage because the encapsulation process may alter antigens and decrease their ability to stimulate the immune system. However, new techniques and types of particles may circumvent this problem.[35] Nanoparticles and microparticles are being tested in companion animals, including horses, as well as in cattle, swine, and fish.[20]

Saponins

Saponins are complex chemical adjuvants extracted from plants, most often the tree *Quillaia saponaria.* The crude extract from this tree is called saponin. Quil A and Spikoside[a] are partially purified mixtures, and QS21 and ISCOPREP 703[b] are defined fractions. Quil A is widely used in veterinary medicine and has been used in vaccines for cattle, pigs, horses, dogs, and cats, including equine influenza virus, canine parvovirus, and FeLV vaccines.[24] QS21 is used in an FeLV vaccine and a canine Lyme disease

vaccine. Saponins are immunomodulators and can induce strong Th1 and Th2 responses as well as CTLs.[9,10,15] They generally are thought to be safe, but their relative safety may depend on the route of administration, the species, and the specific saponin.[10,23,37] IV injections of less purified fractions can result in toxicity, probably due to hemolysis.[23] Injections of free Quil A are well tolerated in sheep and cattle, but some toxicity has been reported in cats.[37] Local inflammatory reactions occur with free Quil A but can be suppressed without loss of adjuvant activity when it is combined with cholesterol-containing liposomes.[23] Purified saponin fractions have a much lower toxicity than Quil A and are being considered for vaccines in humans.[10,23,37]

Immune-Stimulating Complexes

Immune-stimulating complexes (ISCOMs) are cagelike structures that contain saponins, cholesterol, and phospholipids. In veterinary vaccines, the saponin sometimes is Quil A, but more purified fractions are used in vaccines for humans. ISCOMs are immunomodulators.[7,18] They can induce Th1 reactions and CTLs as well as concurrent Th2 responses in some circumstances.[37,38] ISCOMs can be effective adjuvants in cats, dogs, cattle, horses, pigs, sheep, turkeys, rabbits, guinea pigs, and mice.[18,37] They have been used with more than 20 viral, bacterial, and parasitic pathogens and are found in experimental bovine viral diarrhea virus, bovine herpes virus type 1, rinderpest, FeLV, pseudorabies, and canine distemper vaccines.[18,23,38–44] An equine influenza vaccine with an ISCOM adjuvant currently is marketed for horses in Europe. ISCOMs also are being tested for use in vaccines for humans. ISCOMs sometimes have toxic effects in rats and mice, but few adverse effects have been observed in species of veterinary importance.[10,37] Most likely, this observation is due to a dose effect.

Nonionic Block Copolymers

Nonionic block copolymers are synthetic adjuvants composed of blocks of hydrophobic polyoxypropylene flanked by blocks of polyoxyethylene. Nonionic block copolymers are used in shampoos, mouthwashes, and cosmetics generally and are regarded as safe.[45] As adjuvants, these chemicals can enhance humoral immunity to a number of viral, bacterial, and parasitic antigens.[45] They may induce CTLs.[45] Most often, nonionic block copolymers are used in an aqueous buffer and in oil-in-water or water-in-oil emulsions. They are found in combination adjuvants such as Syntex adjuvant formulation (SAF)[c] and IDEC antigen formulation[d] emulsions. These adjuvants may act as immunomodulators, but they mainly appear to improve antigen presentation.[10] They are not biodegradable and can cause local reactions.[10]

Derivatized Polysaccharides

High-molecular-weight sulfated dextrans and DEAE-dextran sometimes are used as veterinary adjuvants. They also have been suggested for use in vaccines for humans. Derivatized polysaccharides may work by saturating Kupffer cells in the liver and preventing antigen degradation.[10]

Carrier Proteins

Protein carriers can be linked to antigens to improve their immunogenicity. Such carriers are especially effective for haptens and carbohydrate antigens, which are poorly immunogenic, especially in the young. Carriers that have been used include diphtheria or tetanus toxoid, KLH, and bovine serum albumin. The toxoids are commonly used for carbohydrate vaccines in humans.[6] Peptides that have been successfully conjugated with KLH to induce an immune response include fragments from bovine papillomavirus-4, tick-borne encephalitis virus, and porcine parvovirus.[46]

Bacterial Products and Their Derivatives

Historically, whole heat-killed bacterial preparations sometimes were used as crude adjuvants. The most famous example is the use of mycobacteria in FCA. More recently, Siwicki et al[47] found that heat-killed lyophilized preparations of *Propionibacterium avidum* KP-40 could enhance the antibody response to a coadministered antigen. For use in modern vaccines, such bacterial preparations must be further refined and often detoxified.

Muramyl dipeptide (MDP) is the active component of an immunomodulatory peptidoglycan from mycobacteria. MDP has important adverse effects, including fever, arthritis, and uveitis, but less toxic derivatives have been made.[6,33] The hydrophilic derivatives (eg, threonyl-MDP, murametide, murabutide, nor-MDP, and *N*-acetylglucosaminyl-MDP) mainly induce Th2 responses.[10] Lipophilic derivatives (eg, MTP-PE [muramyl tripeptide phosphatidyl ethanolamine]) tend to induce Th1 reactions.[10] MDP derivatives often are incorporated into liposomes or into water-in-oil and oil-in-water emulsions. Threonyl-MDP also has been used in experimental FeLV vaccines.[24]

Bacterial toxins also can act as adjuvants. Several adenosine diphosphate (ADP)-ribosylating toxins are being considered for mucosal or transcutaneous use. The 2 that have been tested most extensively are cholera toxin and *Escherichia coli* heat-labile exotoxin (LT). These 2 toxins are promising mucosal adjuvants in some animal models and have been proposed for use in humans.[10,11,15] They appear to induce strong humoral responses as well as CTLs.[11,17] For mucosal use, both LT and cholera toxins must be mutated to less toxic forms, but Glenn et al[48] have discovered that the virulent native toxins are effective adjuvants for transcutaneous immunization. When intact cholera toxin was applied to the skin of mice, immune responses were induced to coadministered antigens with no major adverse effects. Secondary humoral immune responses have been seen in these experiments, and mice were protected against systemic challenge.

Lipopolysaccharide components also can be effective adjuvants. Lipid A and its derivative, 4′ monophosphoryl lipid A (MPL), are immunomodulators that can induce strong Th1 responses.[10] Although lipid A is too toxic to use as an adjuvant, MPL from *Salmonella minnesota* is being considered for use in vaccines in humans.

Gliding bacterial adjuvant (GBA) is a particularly interesting new adjuvant. GBA is a large polymer of amino sugars from the bacterial genus *Cytophaga.* This polysaccharide can stimulate cytokine release in cats, mice, and

humans and appears to be an effective, safe adjuvant.[49,50] GBA seems to be most effective when it is combined with other adjuvants such as alum or an oil emulsion.[49,50] In cats, GBA combined with an oil-based adjuvant promoted markedly higher antibody responses than either alum or Titermax,[e] a potent water-in-oil adjuvant containing copolymer CRL-8941.[50]

Bacterial DNA can act as an adjuvant and induce cytokine release. CpG oligonucleotides are adjuvants that mimic a bacterial DNA motif that is underrepresented in vertebrate DNA. These oligonucleotides contain a central unmethylated CpG dinucleotide, ideally flanked by two 5′ purines (preferably GpA) and two 3′ pyrimidines (preferably TpC or TpT). This motif is 3–20 times more common in bacterial and viral DNA than in mammalian DNA.[51,52] CpG oligonucleotides are immunomodulators, can induce antibodies, and appear to be particularly effective in shifting immunity toward Th1 responses.[51–53] Some authors believe that it may be possible to control the balance between humoral immunity and CMI by titering the concentration of a CpG adjuvant.[53] The safety of these adjuvants remains to be determined. Although low doses appear to be safe in some studies, repeated high doses of CpG oligonucleotides can induce splenomegaly in mice, and bacterial DNA can cause cytokine release and fatal shock.[51,54,55] It may be possible to reduce these adverse effects by incorporating CpG sequences into the sequence of DNA vaccines or by tethering the oligonucleotides to the antigen.[53]

Cytokines

Cytokine proteins and genes themselves are being considered vaccine adjuvants. The specific effects vary with the cytokine: some enhance the activity of defined immune cells, whereas others act as general activators. Cytokines also induce other cytokines, and this property can make the effects of a specific cytokine difficult to predict. Cytokines being considered adjuvants include INF-γ, IL-1, IL-2, granulocyte-macrophage colony stimulating factor, and IL-12.[9] In a limited number of experiments, recombinant IL-1 and IL-2 have been promising as adjuvants in sheep and cattle when combined with other adjuvants.[56] Currently, there is special interest in IL-12, which appears to shift the immune response toward Th1 responses. In cats, IL-12 was an effective adjuvant for an experimental feline immunodeficiency virus subunit vaccine, and IL-12 combined with IL-18 was effective in an FeLV DNA vaccine.[57,58] Although exogenous cytokines seem to shift Th1 or Th2 responses in some trials, they do not in others.[22] A possible explanation is that certain antigens induce such strong humoral responses that the adjuvant cannot influence the result.

Several problems remain before cytokines can be incorporated routinely into vaccines. These proteins often are species-specific, and only a limited number of cytokines have been cloned from species of veterinary importance. Furthermore, work with inbred mice does not always translate well to outbred species of veterinary importance. For example, IL-10 in mice can shift the balance of Th1 and Th2 responses, but in cattle, IL-10 does not have the same effect.[22] Some cytokines may not be stable enough for vaccines. Toxicity also is a concern. Most cytokines are made only in small quantities during the immune response and mainly act locally. When large amounts enter the systemic circulation, the potential for severe shock and death, or less severe adverse effects, exists. Some cytokines also may promote autoimmunity, and overdoses of IL-2 have been linked to autoimmune diseases.[22] An optimal dose can be found for some cytokines, with smaller doses ineffective and larger doses toxic or even immunosuppressive.[22] For other cytokines, the effective dose may be similar to the toxic dose. To overcome some of these difficulties, modified, less toxic derivatives of IL-1 and IL-6 are being developed.[22] An alternative approach is to decrease toxicity by means of cytokine inducers such as avridine, GBA, and MPL.[10,22,49,50]

Complement Derivatives

Components of the mammalian complement system also appear to be promising adjuvants for inducing antibody responses. Fragments of these proteins bind foreign antigens, tagging them for the receptors of antibodies and immune cells. The fragment C3d may be a particularly useful adjuvant. In one study, attaching 3 C3d molecules to an antigen increased its immunogenicity 1,000-fold.[59] Antigens that have been successfully modified by C3d include the influenza virus hemagglutinin, anti-idiotype antibodies, and capsular polysaccharides.[60–62] The fragment C3b also has shown adjuvant activity.[63] One potential concern with complement adjuvants is the possibility that they might activate B cells nonspecifically and induce autoimmunity.[62] To date, this does not seem to have occurred, and the antibody response appears to be specific to the antigen.[62]

Combined Adjuvants

Although some adjuvants such as alum commonly are used alone, many well-known vaccine adjuvants are combination adjuvants. FCA is a water-in-oil emulsion with mycobacteria. The Ciba-Geigy adjuvant formulation is a modified, less toxic version of Freund's adjuvant that incorporates a metabolizable oil (squalene) and nor-MDP.[10] SAF contains a nonionic block copolymer (L121) with threonyl-MDP in squalene, and Ribi DETOX adjuvant[f] consists of MPL and cell wall skeleton in squalene.[33,64]

The result of combining adjuvants depends on the mechanism of action and toxicity of each individual component. Combinations may be better, similar to, or worse than the individual components. Weeratna et al[65] compared adjuvant strength in mice immunized IM with hepatitis B antigen. When single adjuvants were tested, the most effective adjuvants were FCA and Titermax Gold (a water-in-oil adjuvant containing the copolymer CRL-8300), but these 2 adjuvants administered singly also resulted in the most damage to injected muscles. FIA, CpG oligonucleotides, or MPL induced lower titers. FIA caused moderate muscle damage, and CpG oligonucleotides or MPL resulted in mild damage. The least effective adjuvant, with the least amount of damage, was alum. Combining CpG oligonucleotides with alum increased antibody titers to those seen with FCA with minimal muscle damage. CpG oligonucleotides and FIA also resulted in high titers but more muscle damage. Other combinations did not improve efficacy: CpG oligo-

nucleotides and MPL were no more effective than either adjuvant alone. Finally, combining MPL and alum actually reduced the immune response, possibly from toxic effects or interference between their mechanisms of action.

Adjuvants for Mucosal Vaccines

Mucosal vaccines can have marked advantages over systemic vaccines. The benefits may include decreased adverse effects, easier administration, and induction of immunity at the natural point of entry for a pathogen. The choice of adjuvant can be an important factor in the success of these vaccines. The adjuvant must be able to survive harsh conditions, particularly in the gastrointestinal tract. It also is helpful if the adjuvant can protect the antigen and deliver it to local immune tissues. Furthermore, the adjuvant-antigen combination optimally should induce IgA antibodies on mucosal surfaces as well as systemic immune responses. Candidate adjuvants for mucosal vaccines include liposomes, microparticles and nanoparticles, cytokines, ISCOMs, monophosphoryl lipid A, CpG, and detoxified ADP-ribosylating toxins.[6,11,23,38,66,67] Microparticles and nanoparticles are particularly interesting because they may be uniquely able to protect antigens from low pH, bile salts, and enzyme activities.[35] Adjuvants that are able to induce systemic Th1 or CTL responses as well as systemic and mucosal antibodies include microparticles and nanoparticles, ISCOMs, and monophosphoryl lipid A.[6,10,11,18]

Conclusion

Interest in alternatives to oil emulsions and alum has led to the increasing availability of new adjuvants over the last decade. The new generation of adjuvants represents a great diversity of chemical compounds with different mechanisms of action and different potential adverse effects. One goal in adjuvant research has been to find more effective adjuvants with fewer adverse effects. In addition, interest in fine-tuning immune responses has resulted in adjuvants that direct the immune response toward specific types of immunity. Currently, a number of new adjuvants are in clinical trials or available in new vaccines. To select an adjuvant for a vaccine, it must be understood that the best adjuvant is not always the same for all antigens and situations. The optimal adjuvant depends on the animal species, specific pathogen, vaccine antigen, route of immunization, and type of immunity needed. This complexity can be illustrated by the vaccination of cattle against *Streptococcus bovis* and *Lactobacillus* spp. to decrease lactic acidosis.[68] DEAE-dextran with mineral oil, Quil A, and alum all proved better adjuvants than FCA in this situation. Good adjuvants for vaccines against extracellular bacteria will not necessarily be good adjuvants for intracellular pathogens. Mucosal adjuvants may require specific characteristics. Often, adjuvants may need to be tailored to the species as well. Cats appear to be particularly sensitive to inflammation and susceptible to vaccine-induced sarcomas. In this species, a higher priority may need to be placed on less reactive adjuvants. Given these considerations, it should be increasingly possible to design and select adjuvants tailored to the specific needs of the antigen, species, and situation.

Footnotes

[a] Spikoside, Iscotec AB, Stockholm, Sweden
[b] ISCOPREP 703, Iscotec AB, Stockholm, Sweden
[c] SAF, Biocine Co, Emeryville, CA
[d] Antigen formulation, IDEC Pharmaceutical Corp, San Diego, CA
[e] Titermax adjuvant, CytRx Corp, Norcross, GA
[f] DETOX adjuvant, Ribi Immunochem Research, Hamilton, MT

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